Substrate Specificity Studies of Aldolase Enzymes for Use in Organic Synthesis

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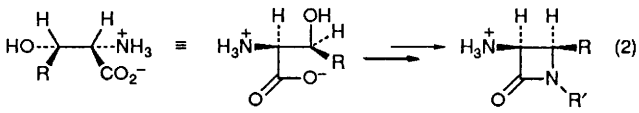
The preparation of β-hydroxy-α-amino acids from glycine and various aldehydes by aldolase enzymes is examined; attention is focused on substrate structural requirements, with stereoselectivity of the aldol reactions also considered.

In recent years, enzymes have found an increasing role in organic synthetic transformations. They are noted for their high degree of stereochemical control and for working under mild conditions to produce compounds often not readily available by traditional synthetic means.[1]

One class of enzymes currently being investigated in this and other laboratories is the aldolases, which includes serinehydroxymethyltransferase[2] (SHMT). Their natural biological roles involve the reversible transfer of one-carbon units to glycine and the retroaldol cleavage of β-hydroxy-α-amino acids such as serine and *allo*-threonine to generate an aldehyde and glycine[3] (equation 1). These enzymes utilize pyridoxal phosphate (PLP) as a cofactor[4] and are derived from many plant, animal, and bacterial sources.[5]

$$HOCHRCH(\overset{+}{N}H_3)CO_2^- \underset{PLP}{\overset{\text{aldolase}}{\rightleftharpoons}} RCHO + \overset{+}{H_3}NCH_2CO_2^- \qquad (1)$$

Such enzymes may be utilized synthetically in the forward aldol manner, *i.e.*, to make β-hydroxy-α-amino acids. These amino acids are of interest for many diverse applications, including use as enzyme inhibitors,[6] components of microbial growth factors, *i.e.*, siderophores,[7] and for preparation of β-lactams[8] (equation 2). A number of preparations of β-hydroxy-α-amino acids exist in the literature, but most have some drawback.[9] These include lack of generality, poor stereochemical control, requirement of chiral auxiliaries, or producing primarily the *threo* (or *syn*) isomers (although examples do exist of *erythro* syntheses[9i–m]). The aldolases have the potential advantage of providing a high degree of both absolute and relative stereochemical control. Furthermore, they form the L-*erythro* isomers, which are not easily accessed from standard chemical aldol reactions. As equation (2) illustrates, the *erythro* (or *anti*) configuration, typical of the enzyme aldol products, is appropriate for eventual conversion to *cis*-substituted β-lactams. The transformation of β-hydroxy-α-amino acids to β-lactams has been studied extensively in this laboratory;[8] thus, it seemed appropriate to explore an enzymatic means of producing the required β-lactam precursors. The enzymes we are studying include those from rabbit liver,[10] corn seedlings,[11] and mung bean seedlings.[12]



(2)

At this point in our studies, we have tested a variety of structures as potential aldehyde substrates for these enzymes.† As part of our overall synthetic strategy, we have sought to determine what kinds of functional groups would be acceptable as components of the aldehydes used. We have also given attention to the effect of the substrate on the position of the equilibrium of the reaction, which for natural substrates favours the retroaldol direction. Table 1 summarizes, in a qualitative form, the results of this work, indicating how well the reactions proceed in the forward (aldol) direction. Substrate processing was monitored by high performance liquid chromatography (HPLC). Figure 1 shows a typical chromatogram depicting product formation in one case.

Table 1. Substrate specificities for aldolases isolated from rabbit liver and corn seedlings.

Entry	R-CHO, R=	Is RCHO a substrate?[a] Rabbit enzyme	Corn enzyme
(1)	-Me	+++	+++
(2)	$-(CH_2)_nMe$	++ $n = 2$	+ $n = 1$
(3)	$-CO_2H$	—	—
(4)	$-(CH_2)_3OTBDMS$[b]	—	Not determined
(5)	$-(CH_2)_2CO_2Me$	+++	++
(6)	$-(CH_2)_2CO_2CH(Me)_2$	+++	Not determined
(7)	$-(CH_2)_2CO_2Me$	+++	++
(8)	-COMe	— (+ with VO_3^- in solution)	— (+++ with tetrahydrofolate in solution)
(9)	$-(CH_2)_3OCHO$	++	Not determined
(10)	-Ph	+++	—
(11)	$-CH_2OCH_2Ph$	Not determined	+
(12)	-2-furyl	+	++
(13)	$-(CH_2)_2$-(2-furyl)	+	—
(14)	-CH=CH-(2-furyl)	—	Not determined

[a] + = detectable substrate, ++ = moderate, +++ = good, — = not a substrate. Incubations assayed by reversed-phase HPLC using fluorescence detection of derivatized amino acids present. Extent of enzyme reaction was determined by peak integration on chromatograms. [b] TBDMS = t-butyldimethylsilyl.

Shown in the table are a number of aldehydes that have been tested with the rabbit liver and corn enzymes. Less work has been done so far with the mung bean enzyme. As seen in the table, a variety of aldehyde structural types is accepted as substrates, including those with either aliphatic or aromatic side chains. Some modest differences in substrate specificities of the two enzymes can also be observed. The rabbit enzyme will accept aliphatic aldehydes quite well, as proven by the rapid processing of aldehydes (**1**), (**2**), (**5**), (**6**), (**7**), and (**9**). The corn aldolase has also been demonstrated to work with some of these compounds [see (**1**), (**2**), (**5**), and (**7**)]. Poor water solubility of the aldehyde is not necessarily a serious problem for reaction with the enzyme, as witnessed by (**2**) ($n = 2$) and (**13**). Aromatic and otherwise unsaturated aldehydes are sometimes also substrates for the enzymes, although usually to a lesser degree (**10**—**14**).

The presence or absence of cofactors can influence the structural specificity of these enzymes. For example, pyruvaldehyde (**8**) is not a substrate for either the rabbit liver or corn enzyme unless a cofactor is present. With the rabbit liver aldolase, inorganic vanadate allows this aldehyde to be processed, possibly *via* the catalytic dehydration of the hydrated aldehyde.[13] Tetrahydrofolate is a cofactor for both enzymes when formaldehyde is a substrate, and the corn enzyme apparently also uses tetrahydrofolate to permit pyruvaldehyde to be converted to an aldol product.

In most cases, diastereoselectivity of the enzyme reaction was good to excellent, based on product peaks observed in chromatograms of the incubation mixtures (see Figure 1). In cases where two peaks were observed, the major diastereoisomer peak was at least an order of magnitude greater than the minor one. The exception to this was benzaldehyde (**10**), which yielded both *threo* and *erythro* diastereoisomers to about the same extent. This was to be expected, since both the *threo* and *erythro* isomers of β-phenylserine are known to be

† Experiments with these enzymes have been done mostly on an analytical scale thus far, generally less than 0.5 ml total volume. The rabbit liver enzyme used was the pure, lyophilized form while the corn seedling enzyme was a partially purified form. For isolation of the corn enzyme, all steps were done at 4 °C. The washed 5—7 day old seedlings (approximately 960 g per litre) were homogenized in a Waring blender in 100 mM potassium monohydrogenphosphate containing 1 mM disodium ethylenediaminetetra-acetate (EDTA), 1 mM dithiothreitol, and 125 μM PLP. The homogenate was filtered through cheesecloth, and the protein which precipitated with 35—50% saturation in ammonium sulphate upon centrifuging at 13 800 × g for 40 min was redissolved in a buffer of 10 mM potassium phosphate, pH 7.8, containing 125 μM PLP and 1 mM EDTA. The sample was then dialysed and concentrated to 5—15 mg protein/ml in the same buffer (except without EDTA). Assay for aldolase activity, measured at this point, consisted of a coupled system between the aldolase-catalysed retroaldol of L-*allo*-threonine with the NADH (reduced form of nicotinamide adenine dinucleotide) dependent reduction of the resulting acetaldehyde by yeast alcohol dehydrogenase (ADH). Specific activity averaged 78 milliunits/mg protein, where one unit of activity is defined as that amount of enzyme required to cause a change of one optical density unit (at 340 nm) per minute at room temperature in the presence of ADH, 120 mM L-*allo*-threonine, 125 μM PLP, and 120 μM NADH in 100 mM phosphate buffer at pH 7.5. The enzyme was generally stored either in lyophilized form or frozen with 10% glycerol. Typical conditions for the incubations are with both the glycine and aldehyde substrates at about 15 mM. Incubations were done at 37 °C, usually in a 10 mM potassium phosphate buffer at a pH of 7.3—8.0 containing 33—80 μM PLP. An enzyme control including all components except the enzyme was incubated alongside its corresponding enzyme-containing sample. Aliquots were removed periodically for analysis. Amino acids present in these samples were monitored by preparing the *o*-phthalaldehyde derivatives[15] and analysing by reversed-phase HPLC with fluorescence detection. HPLC conditions were those outlined in reference 15(b) except that a flow rate of 1.4 ml/min was used and the gradient program (employing two solvent solutions A and B as defined) was altered to the following: 0% B for 3 min from the start of the program, linear step to 14% B in 5 min, isocratic step at 14% B for 5 min, linear step to 100% B in 10 min, linear step to 0% B in 3 min. In cases where authentic material was available [(**1**), (**2**) (where $n = 2$), (**5**) and (**10**)], aldol product formation in the test aliquot was confirmed by HPLC coinjection or comparison of peak retention times (t_R). L-*allo*-threonine, D-*allo*-threonine (t_R 21.5 min), L-threonine, D-threonine (t_R 13.3 min), and DL-*threo*-β-phenylserine (t_R 23.8 min) were obtained commercially. β-Phenylserine (*erythro* t_R 24.7 min) was produced racemically by phase transfer catalytic methods.[17] The aldol product from succinic semialdehyde methyl ester (**5**) (*erythro* t_R 22.5 min) was synthesized asymmetrically as the L-*erythro* diastereoisomer *via* a chiral boron enolate glycine equivalent.[9m] The product derived from butanal (*erythro* t_R 25.5 min, *threo* t_R 24.0 min) was made both in racemic form with copper glycinate[16] and also as the L-*erythro* diastereoisomer by chiral boron enolate chemistry. Sufficient NMR data were collected to ensure the identity of these authentic materials. As an illustration, ^1H NMR data for the *erythro* form of β-hydroxy-α-aminohexanoic acid (in deuterium oxide with sodium 3-trimethylsilylpropionate as reference) are: δ 4.1 (m, 1 H), 3.838 (d, J 3.6 Hz, 1 H), 1.4 (m, 4 H), 0.919 (t, J 6.9 Hz, 3 H).

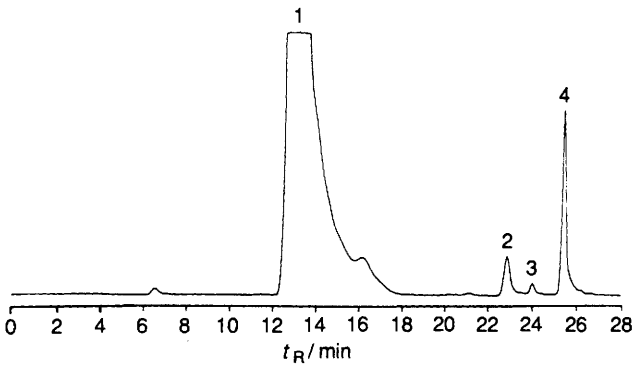


Figure 1. A representative HPLC chromatogram taken from (**2**) in Table 1 (butanal as a substrate for rabbit liver SHMT). The chromatogram shows product formation after 1 h of incubation. The peaks indicated are the *o*-phthalaldehyde derivatives of: (1) glycine, (2) ammonia, (3) minor product diastereoisomer, and (4) the major product diastereoisomer. Peaks 3 and 4 were observed to increase over time, with the major diastereoisomer peak becoming offscale by 4 h of incubation.

substrates for the retroaldol reaction with the rabbit liver enzyme.[14] SHMT is known to be specific for the L-isomers in aldol or retroaldol transformations.[2a,9m] Thus, these enzymes may constitute an effective means of selectively producing the L-*erythro* isomers of β-hydroxy-α-amino acids. In fact, in several cases the identity of the enzyme product was confirmed by comparison with the authentic material obtained by chemical synthetic means. For example, the product derived from butanal was independently prepared in racemic form by aldol condensation of the aldehyde with a copper glycinate chelate.[16] The enzyme product made from the ester (**5**) was shown to be the L-*erythro* diastereoisomer by comparison with authentic material prepared asymmetrically from a chiral boron enolate form of a glycine equivalent.[9m]

In summary, these preliminary results give an indication that the aldolase enzymes have the potential to produce a diverse range of β-hydroxy-α-amino acids from glycine and various aldehydes. Attention is now being directed toward scaling up these reactions to a preparative level, which would allow practical use of the enzyme in asymmetric synthesis.

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